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# Detection of Hydroxylated Nitro Aromatic and Hydroxylated Nitro Polycyclic Aromatic Compounds in an Ambient Air Particulate Extract Using Bioassay-Directed Fractionation

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**16. ABSTRACT** The identification of hydroxylated nitro-polynuclear aromatic hydrocarbons ($OH-NO_2PAHs$) in an ambient air particulate extract by electron impact (EI) and negative chemical ionization (NCI) high resolution gas chromatography/mass spectrometry (HRGC/MS) analyses is presented here. Bioassay-directed fractionation through three sequential separations led to detection of these $OH-NO_2$-PAHs in a highly mutagenic and substantially simplified subfraction of the extract. The compound class identification was based on comparison of both EI and NCI mass spectra of reference $OH-NO_2$-pyrenes. The HRGC/MC retention values for seven $OH-NO_2$-fluoranthene isomer. Quantification of this compound class by NCI HRGC/ MC with on-column injection is demonstrated with sensitivity to 0.5ng/μ L.

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# Detection of Hydroxylated Nitro Aromatic and Hydroxylated Nitro Polycyclic Aromatic Compounds in an Ambient Air Particulate Extract Using Bioassay-Directed Fractionation

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■ The identification and quantification of hydroxylated nitro aromatic compounds in an ambient air particulate extract are presented here. The isomer substitution observed for these compounds supports the mechanism proposed from smog chamber studies: $OH^{\bullet}$ attack on the aromatic compound followed by nitration ortho or para to the OH substituent. Several hydroxylated nitro polycyclic aromatic compounds have been tentatively identified and quantified in the extract also. Both electron ionization (EI) and negative chemical ionization (NCI) high-resolution gas chromatography/mass spectrometry (GC/MS) analyses were used to identify these components. Bioassay-directed fractionation through three sequential separations led to detection of the hydroxylated nitro polycyclic compounds in a mutagenic and substantially simplified subfraction of the extract.

## *Introduction*

Urban air particles contain extractable organic matter that has both mutagenic (*1*) and carcinogenic (*2, 3*) activity. Detection of the specific compounds responsible for the mutagenic activity has been limited by the extremely complex nature of these extracts and the presence of highly polar components that have been difficult to identify. To facilitate identifications, these extracts must be separated into substantially less complex fractions. A single fractionation method followed by microbial mutagenicity studies has been utilized for identifying known or suspected mutagenic compounds in cigarette smoke (*4*), kerosene flames (*5*), synfuels (*6*), xerographic toners (*7*), and diesel exhaust emissions (*8, 9*). Application of this approach, however, to ambient air particulate extracts has to date been unsuccessful in identifying major mutagenic components (*10–12*).

Bioassay-directed fractionation, defined here as sequential fractionation methods of increasing chromatographic resolution, should be the most cost- and time-effective approach for identification of mutagenic compounds in complex mixtures (*13*). Analytical and identification research efforts are directed toward only significantly mutagenic fractions. This approach should be especially useful for the detection of previously unknown constituents in the matrix because the methods are not directed to, or developed for, specific compounds or compound classes.

This paper presents the first mass spectral evidence for detection of hydroxylated nitro polycyclic aromatic hydrocarbons (HN-PAH) and hydroxylated nitro polycyclic aromatic ketones (HN-PAK) in polar fractions of an ambient air particulate extract. To support the proposed existence of these classes of compounds, the confirmed identification of hydroxylated nitro aromatic compounds (HN-AR; AR here for one- and two-ring aromatic compounds) in the same particulate extract is presented. Identified HN-AR include hydroxylated and nitrated benzene, toluene, naphthalene, and biphenyl isomers (HN-Benz, HN-Tol, HN-Naph, HN-Biph). Methyl derivatives of each HN-AR were also tentatively identified.

The rationale and approach by which HN-PAH/PAK were selected as potentially mutagenic species in this extract are also described. Ambient air particulate extracts are clearly too complex for even multiple fractionation steps to provide single-component subfractions. Synthesis and mutagenicity testing cannot be performed for every tentatively identified compound of a mixture. Therefore, a selection method for potentially mutagenic species must be employed. The approach described here uses sequential fractionation to generate multicomponent subfractions and then relies on the separation and identification powers of high-resolution gas chromatography/mass spectrometry (GC/MS) to provide single components for identification. The rationale for selecting the potentially mutagenic species has been based on empirical data: many chemically reactive and mutagenic compounds, e.g., cyclopenta[*cd*]pyrene, benzo[*a*]pyrene, 1-nitropyrene, have higher negative chemical ionization mass spectral (NCI MS) responses relative to their electron ionization mass spectral (EI MS) responses.

Correlation of mutagenic activity with relative NCI/EI MS response has been cited for the isomeric PAH of molecular weight 252 (*14*). In addition, high sensitivity to thermal electron capture, the basis of detection in both electron capture detection GC and NCI MS, has been observed for compound classes that are reactive with macromolecules such as protein and DNA (*15–19*). These observations are consistent with the theory that genotoxic activity is often dependent on interaction of an electrophile, particularly a polycyclic electrophile, with DNA (*20, 21*). Although polycyclic compounds require metabolism to produce the ultimate reactive species, the chemical nature of the original molecule must affect its susceptibility to metabolism. This susceptibility may be due in part to electrophilicity. In the study presented here, comparisons of EI and NCI response from GC/MS analyses have been used to identify potential mutagens.

## *Experimental Methods*

**Chemicals.** To maintain consistency in nomenclature, components will be identified herein as HN-substituted

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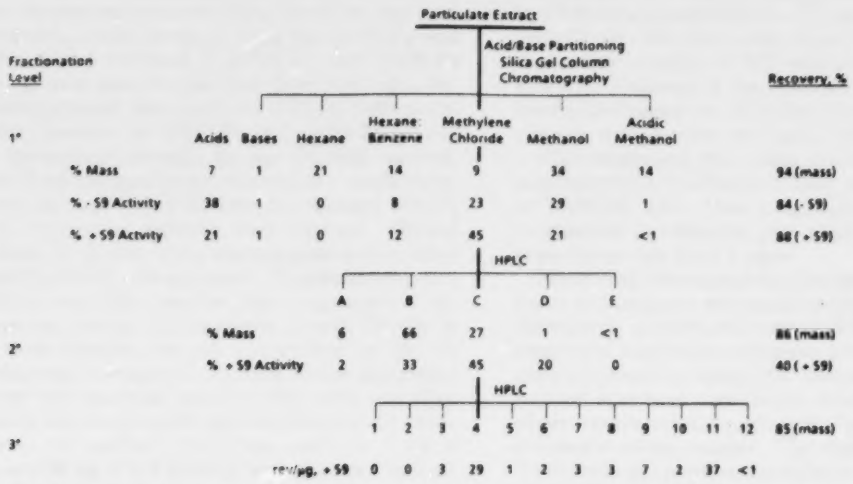


**Figure 1.** Overview of fractionation scheme. Boldface type indicates the pathway leading to analysis of $CH_2Cl_2$-C-4. Distribution and recovery of mass and mutagenic activity calculated by using methods described in ref 27. Subfractions $CH_2Cl_2$-E and $CH_2Cl_2$-C-12 represent laboratory method blanks; HPLC eluant was collected as the solvent program was returned to initial gradient conditions.

**Table I. Source of Reference Hydroxylated Nitro Aromatic Compounds**

| nomenclature used here | conventional nomenclature | source |
|---|---|---|
| 1-OH-2-$NO_2$-benzene | 2-$NO_2$-phenol | Eastman |
| 1-OH-3-$NO_2$-benzene | 3-$NO_2$-phenol | Aldrich |
| 1-OH-4-$NO_2$-benzene | 4-$NO_2$-phenol | Eastman |
| 2-OH-3-$NO_2$-toluene | 6-$NO_2$-2-$CH_3$-phenol | synthesized |
| 2-OH-4-$NO_2$-toluene | 5-$NO_2$-2-$CH_3$-phenol | Pfaltz and Bauer |
| 2-OH-5-$NO_2$-toluene | 4-$NO_2$-2-$CH_3$-phenol | synthesized |
| 2-OH-6-$NO_2$-toluene | 3-$NO_2$-2-$CH_3$-phenol | Aldrich |
| 3-OH-2-$NO_2$-toluene | 2-$NO_2$-3-$CH_3$-phenol | Aldrich |
| 3-OH-4-$NO_2$-toluene | 6-$NO_2$-3-$CH_3$-phenol | Aldrich |
| 3-OH-5-$NO_2$-toluene | 5-$NO_2$-3-$CH_3$-phenol | synthesized |
| 3-OH-6-$NO_2$-toluene | 4-$NO_2$-3-$CH_3$-phenol | Aldrich |
| 4-OH-2-$NO_2$-toluene | 3-$NO_2$-4-$CH_3$-phenol | Aldrich |
| 4-OH-3-$NO_2$-toluene | 2-$NO_2$-4-$CH_3$-phenol | Aldrich |
| 4-OH-3-$NO_2$-biphenyl | 4-OH-3-$NO_2$-biphenyl | Lancaster synthesis |
| 1-OH-2-$NO_2$-naphthalene | 2-$NO_2$-1-naphthol | Analabs |
| 2-OH-1-$NO_2$-naphthalene | 1-$NO_2$-2-naphthol | Tokyo Kasei |

compounds. The thirteen HN-AR obtained from commercial sources are listed in Table I. The three HN-Tol isomers, which are not available commercially, were synthesized according to reported methods (*22–24*).

Reference hydroxylated nitropyrene isomers, 3-, 4-, 5-, 6-, 8-, 9-, and 10-OH-1-$NO_2$-pyrene (e.g., 6-HN-Py), were available from previous studies. The synthesis and characterization of these isomers have been detailed elsewhere (*25*). Reference 2-OH-1-$NO_2$-pyrene (2-HN-Py) was obtained from A. Greenberg, and 2-OH-1-$NO_2$-fluoranthene (2-HN-Fla) was obtained from B. Zielinska. All solvents used were either distilled-in-glass or high-purity grade (Burdick and Jackson).

**Collection, Extraction, and Fractionation.** The air particulate sample was collected in the Philadelphia area over a 2-week period with a massive air volume sampler (MAVS). Collection relies on impaction rather than filtration; the ambient vapor phase is swept past the impacted particles (*26*). Only respirable particles (<1.7 μm), collected on stage III of the MAVS, were used for this study. A side-by-side dichotomous sampler was used to determine the particle concentration in air as a function of size.

A 49.1-g sample of the air particles was extracted in a Soxhlet apparatus under yellow fluorescent lights for 24 h with 300 mL of methylene chloride, for 6 h with a second 300 mL of methylene chloride, and then for 24 h with 300 mL of methanol. The majority of the mutagenicity was in the combined methylene chloride extract (*27*). An overview of the fractionation scheme used is shown in Figure 1. The first fractionation of the extract (level one) involved acid/base partitioning and open-bed silica gel column chromatography (*27*). The elution solvents of the silica gel, used to designate the five fractions collected, ranged from nonpolar hexane to methylene chloride and methanol (*27*).

The second and third fractionations (level two and level three) were achieved with normal-phase high-performance liquid chromatography (HPLC) (Zorbax $SiO_2$, 4.6 mm × 25 cm column, Dupont Instrument Co.). The HPLC system was assembled from modular components consisting of an Altex Model 430 gradient programmer with Model 100A pumps (Altex-Beckman Co.) and an LDC Model 1203 UV detector (Laboratory Data Control Co.). A flow rate of 1 mL/min was used. The eluate was monitored for UV absorbance at 254 nm.

For the level two separation, applied to both the organic acid (Acid) and methylene chloride silica gel eluant ($CH_2Cl_2$), HPLC with a linear gradient of methanol:methylene chloride (2:98, v:v) in hexane was used. The gradient was initiated with 8% of the methanol:methylene chloride mixture and increased to 35% over 30 min. The final composition was held for an additional 30 min. The third subfraction of each to elute, Acid-C and $CH_2Cl_2$-C, with retention of 29–36 min, was analyzed for HN-AR/PAH/PAK.

For the level three separation, applied to $CH_2Cl_2$-C, a linear gradient of 80% methylene chloride in hexane, increasing to 100% methylene chloride in 30 min was used. The final composition was held for 30 min. The fraction in which HN-PAH/PAK were detected, $CH_2Cl_2$-C-4, eluted at 9–14-min retention.

**Distribution and Recovery Studies of HN-Py Isomers.** Recovery and distribution of representative HN-PAH were determined individually for the four separate

fractionation methods used (acid/base partitioning, silica gel column chromatography, and HPLC level two and level three methods). Spike levels of 12 μg for 3-HN-Py and 106 μg of a mixed standard of 6-HN-Py and 8-HN-Py (6/8-HN-Py) were used for the first three methods. Recovery measurements were made by NCI GC/MS as detailed below; because the 6-HN-Py and 8-HN-Py are not resolved chromatographically by the GC/MS method, recovery of these two species are recorded as a single value. For recovery through the HPLC level two method, HN-Py isomers in methylene chloride were injected. Eluant collected from 31–39 min, which encompasses elution times of 3-, 6-, and 8-HN-Py, was analyzed. To estimate recovery in the HPLC level three method, the integrated UV detector response, average of 3 analyses, of each HN-Py in the level three analysis method was divided by the UV detector response, average of 3 analyses, of that compound in the level two analysis method; this ratio was then multiplied by the compound's recovery determined above for the level two method. For these analyses 10 ng of 3-HN-Py and 30 ng of 6/8-HN-Py were analyzed by each HPLC method.

Recoveries of 3-HN-Py (12 μg) and 6/8-HN-Py (106 μg) were determined in which acid/base partitioning was followed by further separation of the acid fraction by the level two fractionation method. Eluant collected from 31 to 39 min was analyzed. The methylene chloride extract of the NBS Standard Reference Material 1649 (Washington, DC Urban Air Particulate Matter) was used as the matrix material.

**Mass Spectrometry Methods.** Low-resolution mass spectra were recorded with a Finnigan Model 4500 mass spectrometer equipped with an electron ionization (EI)/chemical ionization (CI) source. For EI analyses, a 70-eV electron beam, emission current of 0.3 mA, and an ion source temperature of 180 °C were used. For negative chemical ionization (NCI) analyses, methane was used as the reagent gas at an indicated ion source pressure of 1.0 Torr. Methane was ionized by a 150-eV electron beam; the ion source temperature was 180 °C. Data acquisition was controlled by a Finnigan INCOS Model 2100 data system. A scan rate of 1 s was used for both the EI and NCI analyses. The mass range scanned was 80–450 amu for the EI analyses and 100–450 amu for the NCI analyses.

High-resolution gas chromatography (GC) for the EI and NCI GC/MS analyses was achieved with a Hewlett-Packard Ultra No. 2 fused silica capillary column (5% phenylmethylsilicone bonded phase, 50 M × 0.31 mm i.d., 0.52 μm film thickness) with methane carrier gas (linear flow velocity of approximately 75 cm/s at 40 °C). On-column injection at 38 °C was used. For the EI GC/MS analyses, the column temperature was held at 38 °C for 2 min, raised to 100 °C in 3 min (~40 °C/min rise and hold), and programmed 100–320 °C at 8 °C/min. For the NCI GC/MS analyses, the column temperature was held at 38 °C for 0.5 min, raised to 150 °C in 3 min, and then programmed 150–320 °C at 10 °C/min.

HN-AR were quantified against calibration curves of authentic material with 4-$NO_2$-phenol-$d_4$ as internal standard. Reference HN-Py in recovery studies and the isomeric ambient air component were quantified with 1-$NO_2$-pyrene-$d_9$ (1-N-Py-$d_9$) as internal standard. Internal standards were added prior to GC/MS analysis. All calibration standards covered a 100-fold concentration range; calibration curves were linear with a correlation coefficient greater than 0.998. For the tentatively identified hydroxylated nitronaphthalene and hydroxylated nitrobiphenyl isomers, an estimate of the concentration of each was obtained by using the base peak area ratio of unknown to 1-OH-2-$NO_2$-naphthalene. These estimates were made from EI GC/MS data rather than NCI GC/MS data due to the wide variation in NCI response observed for various HN-AR. Estimate of the concentration of the hydroxylated nitrofluorenone (HN-Flo) was based on direct comparison of molecular ion signal with that of 1-N-Py-$d_9$.

High-resolution NCI mass spectra of 6-HN-Py were acquired with a VG Model 70 SEQ operated at a resolution of 10000 (M/ΔM). Mass assignments, made by computer comparison to reference ions of perfluorokerosene, had mass errors less than 5 ppm.

**Microbial Mutagenicity Methods.** The organic extracts and fractions were assayed for mutagenicity toward *Salmonella typhimurium* strain TA98 with and without exogenous metabolic activation (+S9, –S9) in the Ames plate incorporation assay (*28*). Minor modifications to this protocol have been described in detail elsewhere (*29*). The plates were counted electronically by an Artek Model 880 automatic colony counter. The studies reported here for TA98 included positive controls both with activation (2-aminoanthracene) and without activation (2-nitrofluorene). Nonlinear model slopes were determined from the dose response data (*30*).

Fractions from the level one separation were tested at seven doses, in triplicate, with and without S9. Fractions from the level two separation were tested with S9 at three to nine doses with single plates at each dose. Fractions from the level three separation were tested with S9 at a single dose in the range of 1–50 μg, depending on available sample quantity.

## *Results and Discussion*

**Detection and Quantification in Organic Acid Fraction.** Hydroxylated nitrobenzene, -toluene, -naphthalene, and -biphenyl (HN-Benz, HN-Tol, HN-Naph, and HN-Biph) isomers were identified in the acid fraction of this ambient air particulate extract. Additional methyl derivatives of HN-AR were tentatively identified. Relative intensities of diagnostic fragmentation ions of these compounds detected by EI GC/MS are listed in Table II. With exception of the 1-OH-3-$NO_2$- and 1-OH-4-$NO_2$-Benz, all of the HN-Benz and HN-Tol isomers were resolved chromatographically and showed distinct and diagnostic EI and NCI mass spectra. Both EI and NCI mass spectra and chromatographic retention time were used as criteria to confirm identification of these compounds.

Although both 2-OH-1-$NO_2$- and 1-OH-2-$NO_2$-naphthalene were detected in the NCI GC/MS analyses of the acid fraction, of these two only the 2-OH-1-$NO_2$-naphthalene was detected by EI GC/MS. The second HN-Naph isomer (GC retention relative to 1-N-Py-$d_9$ of 0.705, see Table II), tentatively identified on the basis of its similar EI mass spectrum, may be 1-OH-4-$NO_2$-naphthalene. In particular, the EI mass spectrum of this compound is consistent with spectra of HN-AR that do not have ortho substitution of –OH and –$NO_2$ groups: the M – 16 ion (M – O) is more abundant than the M – 17 ion (M – OH).

The 4-OH-3-$NO_2$-biphenyl isomer was only identified in the more sensitive NCI GC/MS analyses. A more abundant HN-Biph isomer, with GC retention relative to 1-N-Py-$d_9$ of 0.685 (see Table II), which elutes earlier than 4-OH-3-$NO_2$-biphenyl, was tentatively identified from EI GC/MS analyses. This HN-Biph isomer may have ortho substitution of –OH and –$NO_2$ groups. Although the M – 16 ion has greater intensity than the M – 17 ion and would, therefore, tend to argue against ortho substitution, the M – 47 ion of this spectrum (12% relative intensity)

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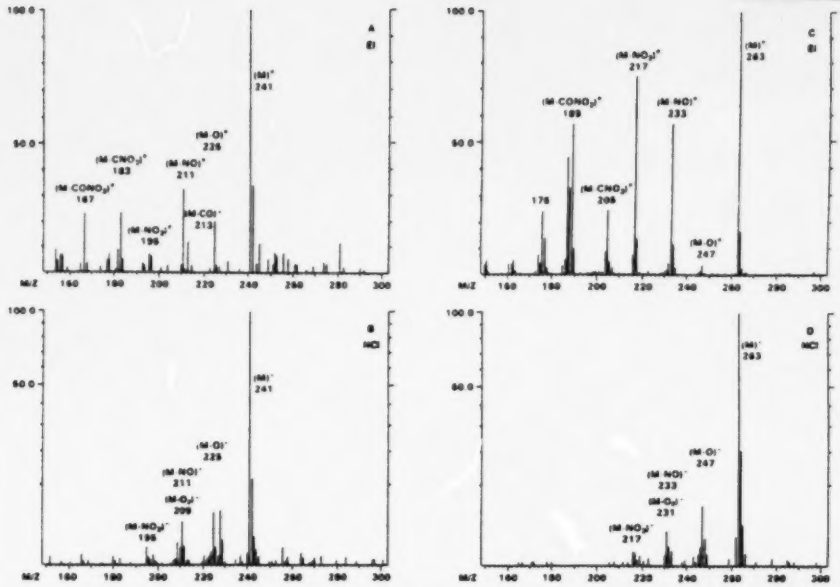


Figure 2. Mass spectra of HN-PAH/PAK. HN-Flo in $CH_2Cl_2$-C-4: EI mass spectrum (A), NCI mass spectrum (B). Reference 6-HN-Py: EI mass spectrum (C), NCI mass spectrum (D). EI mass spectra are displayed with linear intensity scale. NCI mass spectra are displayed with a square root intensity scale to enhance display of low-intensity fragment ions.

is more intense than the M – 46 ion. This latter case, M – 47 ion intensity greater than M – 46 ion intensity, is only observed in ortho-substituted HN-AR, including 1-OH-2-$NO_2$-benzene, 3-OH-2-$NO_2$-toluene, and 4-OH-3-$NO_2$-biphenyl. Although isomeric HN-acenaphthenes should also be considered for the identification of this component given the relatively greater rate of atmospheric $OH^\bullet$ reaction of acenaphthene relative to biphenyl, products resulting from ring opening may prevail over HN-acenaphthenes (*31*, *32*).

In addition to these HN-AR, we have tentatively identified hydroxylated nitro polycyclic compounds including a hydroxylated and nitrated isomer of pyrene (HN-202; pyrene $M_r$ 202), hydroxylated nitrofluorenone (HN-Flo), and two hydroxylated nitromethylfluorenone isomers (HN-MFlo) in the acid fraction. The assignment of the HN-Flo identification was based on the close agreement of its EI and NCI mass spectra with EI and NCI mass spectra produced in this laboratory for HN-Py reference standards. The EI and NCI mass spectra of tentatively identified HN-Flo and reference 6-HN-Py are shown in Figure 2. Both EI spectra show an intense molecular ion (base peak) and major fragment ions corresponding to the loss of O, NO, $CNO_2$, and $CONO_2$ (M – 16, M – 30, M – 58, and M – 74 amu, respectively). In addition, the proposed HN-Flo spectrum contains a fragment ion corresponding to the loss of CO (M – 28). The NCI spectra of the HN-Flo isomer and reference 6-HN-Py show intense molecular ions and major fragmentation ions corresponding to the loss of O, NO, $O_2$, and $NO_2$ (M – 16, M – 30, M – 32, and M – 46). The relative intensities of the M – 30 and M – 32 ions, different for these two compounds, appear to be compound and isomer specific; the ratio of intensity of M – 30/M – 32 ions covers a 40-fold range (0.1–4.0) in the NCI spectra of seven HN-Py isomers.

The NCI mass spectrum of the HN-202 isomer is shown in Figure 3. Diagnostic ion current chromatograms for $m/z$ 263, 247, 233, 231, 217, and 201, corresponding to M, M – O, M – NO, M – $O_2$, M – $NO_2$, and M – $ONO_2$, are also shown. The identification of the HN-202 isomer remains problematic. This compound is most likely a

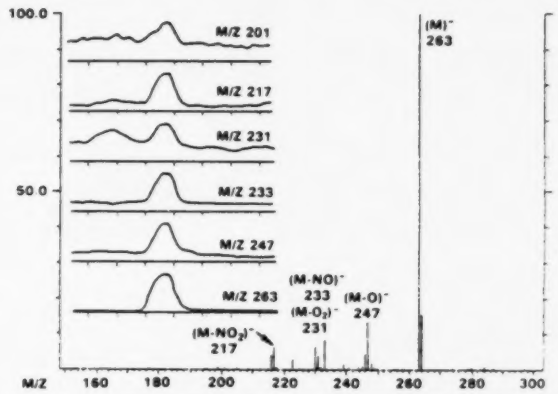


Figure 3. NCI mass spectrum and relevant ion current chromatograms for HN-202 compound detected in ambient air particulate extract.

structural (e.g., HN-fluoranthene/acephenanthrylene or aceanthrylene, all $M_r$ 202 PAH nuclei) rather than positional isomer of HN-Py, as determined by relative retention values.

The retention values of eight HN-Py relative to 1-N-Py-$d_9$ are substantially different from the retention value of this ambient air component (see Table III). The earliest HN-Py to elute was 2-HN-Py, which is least polar by virtue of intramolecular hydrogen bonding. 7-HN-Py, which was not available, would not be expected to elute earlier than 2-HN-Py. Because of the symmetry of the pyrene nucleus, hydroxylation of 2-$NO_2$-pyrene (2-N-Py) and 4-$NO_2$-pyrene (4-N-Py) would result in adjacent and peri carbon atom substituted compounds similar to 2-HN-Py and 10-HN-Py; these isomers should not be substantially less polar than 2-HN-Py.

Table III also includes relative retention values for four $NO_2$-fluoranthene (N-Fla) isomers and two OH-$NO_2$-fluoranthene isomers (HN-Fla) that have adjacent carbon atom substitution (2-OH-1-$NO_2$- and 3-OH-2-$NO_2$-Fla). HN-202 identified here in ambient air elutes earlier than N-Fla or the two HN-Fla.

**Table II. Retention Values and EI MS Ion Intensities of Hydroxylated Nitro Aromatic Compounds Detected in Acid Fraction**

| compound | retention relative to 1-N-Py-$d_9$ | mass (% relative intensity) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | M | M - O | M - OH | M - NO | M - $NO_2$ | M - $CNO_2$ | M - O - $NO_2$ | M - OH - $NO_2$ | M - $CONO_2$ |
| 1-OH-3/4-$NO_2$-benzene | 0.288 | 139 (100) | 123 (21) | 122 (11) | 109 (20) | 93 (49) | 81 (38) | | | |
| 3-OH-6-$NO_2$-toluene | 0.425 | 153 (41) | 137 (11) | 136 (100) | 123 (4) | 107 (10) | 95 (10) | 91 (W)[b] | 90 (21) | [a] |
| 2-OH-5-$NO_2$-toluene | 0.435 | (100) | (15) | (ND)[c] | (29) | (14) | (6) | (W) | (W) | [a] |
| OH-$NO_2$-$C_7$-alkylbenzene | 0.460 | 167 (100) | 151 (16) | 150 (ND) | 137 (25) | 121 (13) | 109 (5) | 105 (W) | 104 (W) | 93 (10) |
| | 0.469 | (80) | (14) | (100) | (W) | (10) | (ND) | (6) | (8) | (8) |
| | 0.483 | (100) | (18) | (50) | (16) | (4) | (W) | (6) | (14) | (6) |
| 2-OH-1-$NO_2$-naphthalene | 0.666 | 189 (56) | 173 (W) | 172 (W) | 159 (W) | 143 (W) | 131 (42) | 127 (W) | 126 (W) | 115 (100)[d] |
| OH-$NO_2$-naphthalene isomer | 0.705 | (100) | (14) | (5) | (23) | (26) | (23) | (ND) | (9) | (89) |
| OH-$NO_2$-methylnaphthalene | 0.693 | 203 (100) | 187 (8) | 186 (24) | 173 (27) | 157 (20) | 145 (14) | 139 (15) | 138 (ND) | 129 (31) |
| OH-$NO_2$-biphenyl/acenaphthene | 0.685 | 215 (100) | 199 (8) | 198 (W) | 185 (14) | 169 (5) | 157 (9) | 153 (W) | 152 (W) | 141 (36) |
| OH-$NO_2$-methylbiphenyl/acenaphthene | 0.810 | 229 (100) | 213 (7) | 212 (ND) | 199 (40) | 183 (24) | 171 (14) | 167 (6) | 166 (5) | 155 (35) |
| | 0.921 | (100) | (24) | (ND) | (36) | (18) | (5) | (20) | (ND) | (56) |

[a] Instrument not scanned low enough to detect this ion. [b] Weak, <4% relative intensity. [c] Not detected. [d] Ions at $m/z$ 89 and 103 also used to confirm identification.

**Table III. Relative Retention Values for Selected $NO_2$-Fluoranthene/$NO_2$-Pyrene Isomers and OH-$NO_2$-Fluoranthene/OH-$NO_2$-Pyrene Isomers**

| compound | retention relative to 1-N-Py-$d_9$ |
|---|---|
| tentatively identified OH-$NO_2$ M, 202 PAH isomer (HN-202) | 0.865 |
| 1-$NO_2$-fluoranthene | 0.944 |
| 7-$NO_2$-fluoranthene | 0.951 |
| 2-$NO_2$-fluoranthene | 0.966 |
| 3-$NO_2$-fluoranthene | 0.971 |
| 2-OH-1-$NO_2$-fluoranthene | 0.991 |
| 8-$NO_2$-fluoranthene | 0.998 |
| 1-$NO_2$-pyrene | 1.002 |
| 3-OH-2-$NO_2$-fluoranthene[a] | 1.008 |
| 2-OH-1-$NO_2$-pyrene | 1.041 |
| 10-OH-1-$NO_2$-pyrene | 1.112 |
| 9-OH-1-$NO_2$-pyrene | 1.203 |
| 4-OH-1-$NO_2$-pyrene | 1.203 |
| 3-OH-1-$NO_2$-pyrene | 1.217 |
| 6-OH-1-$NO_2$-pyrene | 1.232 |
| 8-OH-1-$NO_2$-pyrene | 1.232 |
| 5-OH-1-$NO_2$-pyrene | 1.232 |

[a] Major product formed from NaOH reaction with 2-$NO_2$-3-$NH_2$-fluoranthene and also formed from direct nitration of 3-OH-fluoranthene.

**Table IV. Concentration of HN-AR, HN-PAH, and HN-PAK**

| compound | concentration | |
|---|---|---|
| | extract, μg/g | air, ng/m$^3$[a] |
| 1-OH-3/4-$NO_2$-benzene[b] | 30 | 0.05 |
| 3-OH-6-$NO_2$-toluene | 1900 | 3 |
| 2-OH-5-$NO_2$-toluene | 4800 | 8 |
| 1-OH-2-$NO_2$-naphthalene | 20 | 0.03 |
| 2-OH-1-$NO_2$-naphthalene | 6 | 0.01 |
| OH-$NO_2$-naphthalene isomer[c] | 400[d] | 0.6 |
| 4-OH-3-$NO_2$-biphenyl | 10 | 0.02 |
| OH-$NO_2$-biphenyl isomer | 700[d] | 1 |
| OH-$NO_2$-202 PAH isomer | 5 | 0.01 |
| OH-$NO_2$-fluorenone isomer | 10[d] | 0.02 |

[a] Concentration in air extrapolated from concentration in respirable particles (<1.7 μm) using particle concentration in air. [b] 1-OH-3-$NO_2$-benzene and 1-OH-4-$NO_2$-benzene not resolved chromatographically. [c] May be 1-OH-4-$NO_2$-naphthalene on the basis of EI mass spectrum. [d] Estimate of concentration as explained under Experimental Methods.

Structural isomers such as HN-acephenanthrylenes and HN-aceanthrylenes cannot be excluded as the identification of this unknown. Recent studies have demonstrated gas-phase nitration of acephenanthrylene (*33*); the proposed mechanism, involving OH• attack and ortho nitration, suggests hydroxylated nitroacephenanthrylenes may also be formed. These smog chamber studies combined with the need to identify a compound that is substantially less polar than any HN-Py isomer, possibly through interaction of OH and $NO_2$ groups in a bay region, suggest the possibility of a PAH nuclei other than atmospherically abundant fluoranthene and pyrene for this HN-202.

The identified HN-AR were quantified in the acid fraction of the particulate extract. HN-PAH/PAK were quantified in a subfraction of the acid fraction with HPLC retention of 29–36 min, level two separation method. Extrapolated values for concentrations of HN-AR/PAH/PAK in the methylene chloride extract of the respirable particles and in the air are shown in Table IV. Ambient concentration of species in air is based only on

the concentration of particles <1.7 μm in the site air; for the lower molecular weight species, the vapor-phase component may significantly increase ambient levels.

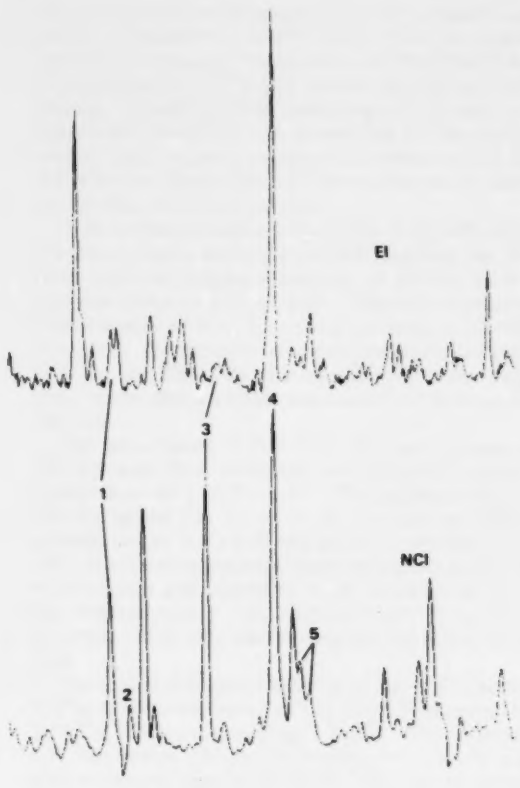
**Bioassay-Directed Fractionation.** Tentatively identified HN-PAH/PAK of Table IV were detected initially in a polar neutral component fraction by use of bioassay-directed fractionation methods as shown schematically in Figure 1. The distribution and recovery of mass and mutagenic activity, using methods described elsewhere (*27*, *34*, *35*), for the level one and level two fractions and the mutagenic activity (revertants/μg) of level three fractions are shown in Figure 1. The mutagenic activities (revertants/μg) for level one fractions are given elsewhere (*27*).

No known mutagens were identified among the phthalate esters, aliphatic aldehydes, aliphatic acid esters, and polycyclic aromatic ketones (*19*) detected in the $CH_2Cl_2$ fraction. Individual peaks in the EI and NCI GC/MS chromatograms of $CH_2Cl_2$-C were relatively broad and contained at least four to six distinct molecular ions with overlapping fragment ion spectra. Major components in the NCI analysis were not found in the EI analysis.

Since mutagenic activities of subfractions $CH_2Cl_2$-C-4 and $CH_2Cl_2$-C-11 (29 and 37 revertants/μg, respectively) were higher than the activity of $CH_2Cl_2$-C (9 revertants/μg), both $CH_2Cl_2$-C-4 and $CH_2Cl_2$-C-11 were analyzed. Most GC/MS peaks of $CH_2Cl_2$-C-4 and $CH_2Cl_2$-C-11 contained a single component, and major components in the NCI analysis were also detected in the EI analysis.

The comparison of EI and NCI data, alluded to above, was used initially to determine whether additional fractionation was required. If individual GC/MS chromatographic peaks contained a single component and the same set of compounds were detected in both EI and NCI analyses, further fractionation would not be pursued. The latter criterion was chosen on the assumption that polar ambient air mutagens, like nitro-PAHs (N-PAH), may be electronegative and present at low concentration levels in the original extract. Because NCI greatly favors ionization and, hence, detection of electronegative compounds, detection of subfraction components by both MS techniques provides reasonable assurance that even the low-level electronegative components of the subfraction have been detected.

Numerous components of $CH_2Cl_2$-C-4 were identified in both EI and NCI GC/MS analyses. Of the tentatively identified HN-PAH/PAK in $CH_2Cl_2$-C-4, only the HN-Flo was detected in both EI and NCI analyses (see Figure 4). These analyses demonstrated realistic rather than rigorous application of the criteria discussed above. Further fractionation of $CH_2Cl_2$-C-4 was not possible due to limited sample quantities. HN-Flo was the second most intense peak in the NCI analysis, 75% relative to the most abundant. The quantified concentration levels of HN-Flo and HN-202 in the acid fraction compare only moderately well with the relative abundances of these two compounds as observed in the NCI GC/MS analysis of $CH_2Cl_2$-C-4. This may be due to quantification of HN-Flo only on the basis of the 1-N-Py-$d_9$ signal, rather than on the basis of a calibration curve of authentic material, or may be due to a greater degree of decomposition for HN-Flo relative to HN-202. Considerable time (>1 year) elapsed between detection of HN-Flo in $CH_2Cl_2$-C-4 and quantification in the subfraction of the acid fraction. A major component of the NCI HRGC/MS analysis of $CH_2Cl_2$-C-11 and minor component of $CH_2Cl_2$-D has been tentatively identified as a hydroxylated nitroazaarene (*36*). The high NCI MS response relative to the EI MS response suggests that this component and the HN-PAH/PAK observed in $CH_2Cl_2$-C-4 may show mutagenic activity.




1 BBP
2 OH-$NO_2$-MW202 PAH
3 OH-$NO_2$–Fluorenone
4 DEHP
5 OH-$NO_2$–Methyl Fluorenone

**Figure 4.** Selected portion of total ion current chromatograms of EI and NCI GC/MS analyses of $CH_2Cl_2$-C-4. BBP (butyl benzyl phthalate) and DEHP (di-2-ethylhexyl phthalate) are laboratory contaminants; phthalates originally present in extract are found in $CH_2Cl_2$-A.

**Distribution and Recovery of HN-Py.** Detection of these HN-PAH/PAK in the HPLC retention windows of $CH_2Cl_2$-C and $CH_2Cl_2$-C-4 is consistent with solubility and polarity of reference HN-Py; four HN-Py (3-, 4-, 9-, and 10-HN-Py) elute with the retention of $CH_2Cl_2$-C, and three HN-Py (4-, 6-, and 9-HN-Py) elute with the retention of $CH_2Cl_2$-C-4.

Detection of HN-PAH/PAK in a polar neutral fraction is consistent with distribution and recovery studies carried out on reference HN-Py; the distribution of the ambient air HN-202 isomer between organic acid and neutral component fractions (99.7 and 0.3%, respectively) compared well with distribution of the reference HN-Py (99.8 and 0.2%). As shown in Table V section (A), a large percentage of HN-Py (>85%) partitioned into the acid fraction, but a quantifiable amount remained in the neutral component fraction. On deactivated silica gel, these HN-Py eluted primarily in the $CH_2Cl_2$ fraction. While recovery of HN-Py from the silica gel HPLC column in which the mobile phase was modified with methanol (level two method) was significantly better, as expected, than recovery from that column without methanol in the mobile phase (level three method), a quantifiable amount was recovered in each step. In section (B) of Table V, recovery results are shown for reagent and matrix spikes at 3-HN-Py and 6/8-HN-Py in

Table V. Distribution and Recovery of Representative HN-PAH in Fractionation Procedures Used

| | % recovery | |
|---|---|---|
| | 3-HN-Py | 6/8-HN-Py |
| (A) Separately Performed Steps–Reagent Spikes (Fractions Analyzed) | | |
| acid/base partitioning[a,b] | | |
| acid fraction | 102 | 86 |
| neutral fraction | 0.2 | 0.2 |
| total recovery | 102 | 86 |
| silica gel column chromatography[a,c] | | |
| hexane:benzene | ND[d] | ND |
| methylene chloride | 12 | 53 |
| methanol | 7 | 5 |
| total recovery | 19 | 58 |
| level two HPLC method[a,e] | | |
| retention window 31–39 min | 73 | 78 |
| level three HPLC method[f] | | |
| retention window 9–25 min | 25 | 52 |
| (B) Combined Steps | | |
| acid/base partitioning and level two HPLC method | | |
| reagent spike[g] | 77 | 71 |
| matrix spike[h] | 60 | 45 |

[a] Duplicate analyses. [b] Spike solution diluted to 50 mL with $CH_2Cl_2$; partitioned 3 times with 40 mL of NaOH and $H_2SO_4$; aqueous phase back-extracted 3 times with 40 mL of $CH_2Cl_2$; 3-HN-Py analyses ±10%; 6/8-HN-Py analyses ±10%. [c] Spike solution solvent exchanged with hexane and placed at head of 10-g silica gel column; column eluted with 80 mL of hexane and 140 mL each of hexane:benzene, methylene chloride, and methanol; 2 mL of each elution solvent, used to rinse reagent spike vessel, was used as initial portion of eluant; 3-HN-Py analyses ±5%; 6/8-HN-Py analyses ±10%. [d] Not detected. [e] Reagent spike concentrated to 100 μL; three analyses (two 50-μL injection of sample and one 50-μL injection of vessel rinse) by HPLC method with pooled collection of retention window listed; analyses ±5%. [f] Recovery estimated as ratio of HPLC UV response for triplicate analyses of standards with each HPLC gradient, multiplied by recovery of preceeding step. [g] Acid/base partitioning performed as in footnote *b*, and acid fraction separated by HPLC as in footnote *e*. [h] Spike solution added to 50 mg of methylene chloride extract of NBS Standard Reference Material 1649 (Washington, DC, Urban Air Particulate matter); sample separated as in footnote *g*.

which acid/base partitioning was followed by isolation of acid fraction HN-Py with the level two fractionation method. By this procedure, recovery of the HN-Py isomers was slightly higher than 70% for the reagent spikes and averaged 50% for the matrix spikes.

## *Conclusions*

The presence of HN-PAH in particulate matter has been suggested by Lõfroth, Neilsen, and Schuetzle to account for mutagenicity in extract fractions more polar than those containing N-PAH (*37–39*). Mechanisms have been proposed by which atmospheric hydroxylation of PAH followed by ortho and para nitration readily yields both N-PAH and HN-PAH (*38–40*). 1-OH-2-$NO_2$-naphthalene, 2-OH-1-$NO_2$-naphthalene, and 4-OH-3-$NO_2$-biphenyl reported here support the ortho nitration mechanism. 3-OH-6-$NO_2$-toluene and 2-OH-5-$NO_2$-toluene would arise from nitration para to OH on *m*-cresol and *o*-cresol, respectively.

Recently, HN-Fla isomers have been produced from reaction of Fla with $N_2O_5$ and $N_2O_5/HNO_3$ mixtures in $CCl_4$ solution at ambient temperature (*41*). Despite detection in these studies, HN-PAH have not been identified in ambient air, largely due to the complexity of the particulate extracts. Mutagenic components of organic acid fractions have not been reported. The data here represent the initial step in demonstrating the existence and potential mutagenicity of HN-PAH/PAK in ambient air particulate extracts. The detection of HN-PAH/PAK, and in particular of a HN-202 isomer, should not be unexpected. Several N-PAH, including 1-N-Py and 2-N-Fla, have been identified and quantified in this particulate extract [(*42*); recent analyses with reference 2-N-Fla and 3-N-Fla have shown that 2-N-Fla was incorrectly identified as 3-N-Fla in this reference].

With a tandem mass spectrometric (MS/MS) technique the presence of a molecular ion and fragment ion (M – 16) were used to propose detection of HN-Py in a diesel particle emission extract (*39*). Schuetzle estimated the concentration of HN-Py to be in the range of 10–100 μg/g of extract. On the basis of the mutagenicity of a mixture of 3-, 6-, and 8-HN-Py, the contribution to the mutagenicity of one diesel extract was estimated to be as high as 5% (*43*).

The importance of HN-PAH became apparent when HN-Py were first identified as mutagenic mammalian metabolites of 1-N-Py (*25*). The mutagenicity of the HN-Py tested (3-, 4-, 5-, 6-, 8-, 9-, and 10-OH-1-$NO_2$-pyrene) ranged from 0.06 to 8 times the activity of 1-N-Py (*44*). HN-Py were generally more mutagenic in the absence of activation and appeared to be metabolized in vivo to the corresponding ring-hydroxylated N-acetylaminopyrenes, which were also mutagenic but required activation.

The higher mutagenic activity of the N-Fla isomers [8-N-Fla, 42000 revertants/μg; 3-N-Fla, 32000 revertants/μg; 2-N-Fla, 4000 revertants/μg; 1-N-Fla, 1900 revertants/μg (*45*, *46*)] versus 1-N-Py [1000 revertants/μg (*7*)] suggests that structural isomers of HN-Py may also be mutagenic. Confirmation of the structure of these tentatively identified HN-PAH/PAK and detection of them in other representative ambient air samples are being pursued. These data underscore the importance of further synthetic work and elucidation of the potential health effects of HN-PAH/PAK.

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We acknowledge helpful editorial and technical discussions with S. V. Lucas (Battelle Columbus Division) and laboratory assistance of C. C. Chuang and W. D. Vermillion (Battelle Columbus Division) and A. Austin (U.S. EPA). We also acknowledge the gifts of 2-OH-1-$NO_2$-pyrene from A. Greenberg (New Jersey Institute of Technology, Newark, NJ) and 2-OH-1-$NO_2$-fluoranthene from B. Zielinska (University of California at Riverside, Riverside CA).

**Registry No.** 3-HN-Py, 86674-49-9; 6-HN-Py, 1767-28-8; 8-HN-Py, 1732-29-2; 1-OH-3-$NO_2$-Benz, 554-84-7; 1-OH-4-$NO_2$-Benz, 100-02-7; 3-OH-6-$NO_2$-Tol, 2581-34-2; 2-OH-5-$NO_2$-Tol, 99-53-6; 2-OH-1-$NO_2$-Naph, 550-60-7; OH-$NO_2$-Naph, 82322-43-8; OH-$NO_2$-methyl-Naph, 114184-74-6; OH-$NO_2$-Biph, 95443-98-4; OH-$NO_2$-acenaphthene, 100441-33-6; OH-$NO_2$-methyl-Biph, 114184-75-7; OH-$NO_2$-methyl-acenaphthene, 114184-76-8.

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Additions/Corrections

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Pg. 910, col. 1, line 11 from the bottom: HN-AR were quantified in separate analyses against calibration curves of authentic material with either 1-$NO_2$-pyrene-$d_9$, 2,4-dimethylphenol-3,5,6-$d_3$ or 4-$NO_2$-phenol-$d_4$ as internal standard.
Pg. 910, col. 2, line 28 from the bottom: The HN-3enz and HN-Tol isomers were resolved chromatographically (2-OH-4-NO2-Tol and 3-OH-5-NO2-Tol resolved at a temperature program rate of 6 °C/min), etc.

Table II:
1-OH-3-$NO_2$-benzene, RRT=0.384 139(100); 123(21); 122(ND); 109(20); 93(49);81(47)
1-OH-4-$NO_2$-benzene, RRT=0.395 139(100); 123(19); 122(ND); 109(36); 93(23); 81(20)[e]
[e] 1-OH-2-$NO_2$-benzene tentatively identified on basis of relative retention as component with RRT=0.288

Table IV:
1-OH-3-NO2-benzene, concentration in extract 30 µg/g, concentration in air 0.05 ng/$m^3$
1-OH-4-NO2-benzene, concentration in extract 470 µg/g, concentration in air 0.8 ng/$m^3$.
Delete footnote (b)